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UPTAKE OF T-2 MYCOTOXIN IN CULTURED CELLS. RELATIONSHIP TO SODIUM FLUORIDE AND CELL TYPE.

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was affected by both temperature and sodium fluoride. At 37°C, toxin uptake plateaued by 30 min in the presence of sodium fluoride. At 22°C, the rate of toxin uptake was slower with or without sodium fluoride present. Statistical analysis of individual time points along the curve demonstrated that sodium fluoride significantly increased cell-associated toxin at most time points. Analysis of the slopes of uptake curves from 0 to 20 min indicated significant ($p<0.05$) differences in the rates of T-2 uptake in both cell types and toxin doses in the presence of sodium fluoride. The increase in toxin uptake in the presence of sodium fluoride was not due to altered cell membrane permeability caused by sodium fluoride. Difference in total uptake between cell types may reflect cell specific factors such as the number of intracellular binding sites. This study demonstrates that sodium fluoride significantly increases cell-associated T-2 toxin and the race of toxin uptake in two cultured cell lines. Of the various actions of sodium fluoride, inhibition of ATP produced by glycolysis may be the most important. Sodium fluoride induced inhibition of glycolytic energy production may lead to increased cellular uptake of T-2, decreased cellular export of T-2, or a combination of both events.

ABSTRACT

TRUSAL, L. R. Uptake of T-2 toxin in cultured cells. Relationship to sodium fluoride and cell type. Toxicon __, _____ _____, 19 ___.

We examined the effect of sodium fluoride on uptake of tritium-labeled T-2 toxin (molecules/cell) in Chinese hamster ovary (CHO) and African green monkey kidney (VERO) cells. Correlations were made to temperature (22, 37° C), and toxin concentration (0.001 and 0.01 µg/ml) over time (0-180 min). As expected, toxin uptake increased in both cell types with increasing time and temperature. Under all parameters, VERO cells exhibited significant ($p<0.5$) increases in the rate of toxin uptake. At each toxin dose, the rate of toxin uptake in both cell types was generally greater at 37° C compared to 22° C. The rate of equilibrium was affected by both temperature and sodium fluoride. At 37° C, toxin uptake plateaued by 30 min in the presence of sodium fluoride. At 22°C, the rate of toxin uptake was slower with or without sodium fluoride present. Statistical analysis of individual time points along the curve demonstrated that sodium fluoride significantly increased cell-associated toxin at most time points. Analysis of the slopes of uptake curves from 0 to 20 min indicated significant ($p<0.05$) differences in the rates of T-2 uptake in both cell types and toxin doses in the presence of sodium fluoride. The increase in toxin uptake in the presence of sodium fluoride was not due to altered cell membrane permeability caused by sodium fluoride. Difference in total uptake between cell types may reflect cell specific factors such as the number of intracellular binding sites. This study demonstrates that sodium fluoride significantly increases cell-associated T-2 toxin and the rate of toxin uptake in two cultured cell lines. Of the various actions of sodium fluoride, inhibition of ATP produced by glycolysis may be the most important. Sodium fluoride induced inhibition of glycolytic energy production may lead to increased cellular uptake of T-2, decreased cellular export of T-2, or a combination of both events.

INTRODUCTION

T-2 mycotoxin is a small, non-protein toxin produced by several Fusarium species (UENO, 1977). Although T-2 toxin has been studied for many years as an agricultural grain contaminate (LUTSKY et al., 1978), it has gained increased interest because of its suspected use in military conflicts (HAIG, 1982; MIROCHA et al., 1982). Its principle mode of action in both in vivo and in vitro systems is as a potent inhibitor of eucaryotic protein synthesis (McLAUGHLIN et al., 1977). Although the intercellular site of action is considered to be the 60S ribosomal subunit (CANNON et al., 1976; HOBDEN and CUNDLIFFE, 1980), little has been published about the method of binding, uptake or internalization of the toxin.

Sodium fluoride has been shown to inhibit cell growth and be cytotoxic with prolonged exposure (HOLLAND, 1980). In various cell lines, fluoride causes inhibition of protein synthesis, which is believed to be the primary cause of its cytotoxicity (HOLLAND et al., 1980, MANKOVITZ et al., 1978) In addition, fluoride exposure results in an enolase-mediated inhibition of glycolysis (CIMASONI, 1972).

Preliminary studies indicated that various chemicals affected uptake of T-2 toxin in cultured cells. Since sodium fluoride had the largest effect, we studied its effect on uptake of T-2 toxin in cells lines used for mycotoxin studies in our laboratory. Parameters of toxin concentration and cell type were correlated with toxin uptake over time. In addition, such factors as ribosomal content and cell volume were investigated as they related to the results.

MATERIALS AND METHODS

Cultured cells Chinese hamster ovary (CHO) and African green monkey kidney (VERO) cells were added to 24-well dishes (Costar, Cambridge, MA) at a density of 1.0×10^5 cells/well and allowed to reach confluency overnight in a 37° C warm box. Culture medium consisted of Hanks 199 (H-199) (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum, 25 mM HEPES buffer, penicillin (100 units/ml) and streptomycin (100 µg/ml).

Toxin uptake assay Tritium-labeled T-2 toxin (Amersham, Arlington Heights, IL)(specific activity 14 Ci/mmol), was added to media just prior to use. Radiolabeled toxin was added to 6 wells for each temperature, concentration, and time point. At designated times, toxin was removed and cells rinsed with Hanks balanced salt solution (HBSS). The monolayer was digested with 0.1 M NaOH (1.0 ml), added to aquasol (New England Nuclear, Boston, MA) (9.0 ml), and counted in a liquid scintillation counter (Mark II Scintillation Counter, Searle Analytic, Inc., Des Plaines, IL). Cell counts were performed on 12 duplicate wells by using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL). Average cell counts were used to calculate molecules of T-2 taken up per cell, based on the specific activity of the toxin.

Drug experiments Sodium fluoride was dissolved in water (1.0 M), and added to appropriate wells at a final concentration of 5 mM. Cells were preincubated with sodium fluoride for 1 hr, after which time, [^{3}H]-T-2 (0.01 μg/ml) was added for time periods ranging from 0 to 180 min at 30 min intervals. Time periods used for studies of the slope of initial toxin uptake curves ranged from 0 to 20 min. A reverse time sequence was used so that all wells could be processed simulataneously as previously described.

Effect of sodium fluoride To determine the effect of sodium fluoride in our system, we exposed CHO and VERO cultures in 24-well dishes to 5 mM sodium fluoride for various time periods ranging from 0 to 240 min at 30 min intervals. Culture media was then assayed for release of lactate dehydrogenase (LDH) (EC 1.1.1.27) expressed in international units per liter (IU/l) which represent the mean of 3 replicate determinations. Background LDH levels in culture media were subtracted from each time point as a control (HARMAN AND FISCHER, 1982). The LDH assay was performed on a COBAS BIO (Roche Analytical Diagnostic Systems, Nutley, NJ).

Ribosomal determinations Ribosomal content of both CHO and VERO cells was estimated by determining total cellular RNA content of 50 cells of each type. Monolayer cultures used for RNA cytophotometry were treated with DNase and stained by SHEA's (1970) modification of the azure-B histochemical procedure of FLAX AND HIMES (1952.) Total cellular RNA content was then quantified using a Vickers M85 scanning-integrating microdensitometer (Vickers Instruments, Malden, MA). This instrument provides a readout of the integrated extinction of azure B-RNA content in absorbancy units (A.U.). Cytophotometric measurements were determined at 580 nm, which was predetermined to be the maximum absorbance of the azure B-RNA complex. Ribosomal RNA content was then estimated to be 80% of the total cellular RNA content according to WATSON (1976).

Cell volume determinations Cell volume for CHO and VERO cells were estimated using by using tritiated water method. Cells were added to 12 wells of a 24-well dish, at a density of 1.5×10^5 cells/well and incubated overnight. Identical wells were set up for cell counts. Media was removed and cells rinsed with HBSS. HBSS containing 10% FCS and hepes buffer was then added for 1 hr at 37° C followed by 10 μl of [^{3}H] water (3 μCi/ml) (New England Nuclear, Boston, MA) for 3 hr. HBSS was removed and saved for scintillation counting. Each well was quickly rinsed to remove non-cell-associated radiolabel and cells were lysed as previously described. Equal volumes (1 ml) of HBSS and NaOH were added to separate volumes of aquasol-2 (9 ml) and counted by liquid scintillation. Cell volume was determined by dividing radiolabeled counts of the HBSS sample by radiolabeled counts of the cell lysate. This figure was divided by the average cell count to yield a relative cell volume figure. Ratios of VERO/CHO cell volume were calculated and represented the mean of 2 experiments.

Statistics Statistical analysis of the slope of the uptake curves was performed by using an analysis of covariance. Significance of dose (covariate), temperature and cell type were determined. Finally, multiple comparisons of the slopes (adjusted for standard errors) were made among the various cell type and temperature combinations by the Tukey-Kramer test (STOLINE, 1981; KRAMER, 1956, 1970) with a significance difference confidence level of $p<0.05$. Total cellular RNA values by using the Duncan Multiple Range Test with a significant difference confidence level of $p<0.05$. Data on the effect of sodium fluoride on LDH release were analyzed by a one-way analysis of variance.

RESULTS

The exposure to 5 mM sodium fluoride for up to 4 hr was not cytotoxic to either CHO or VERO cell lines as assayed by release of lactate dehydrogenase. Results are shown in Table 1.

Results of T-2 toxin uptake in CHO and VERO cells at 22 and 37^0 C in the presence and absence of sodium fluoride are illustrated in Figures 1 A-D. In both cell types, toxin uptake increased significantly with increasing time and temperature. Sodium fluoride also caused significant increases in toxin uptake in both cell types. At a T-2 dose of 0.001 µg/ml, sodium fluoride significantly ($p<0.05$) increased toxin uptake in both CHO and VERO cells at all time points and at both temperatures (Figs. 1A and 1C). At 0.01 µg/ml, sodium fluoride increased T-2 uptake in both cell types and at both temperatures in 19 out of 24 time points (Figs. 1B and 1D). Under all parameters tested, sodium fluoride significantly ($p<0.05$) increased toxin uptake in VERO cells compared to CHO cells at all time points (Figs 1 A-D). At 0.01 µg/ml, after 90 min, T-2 toxin uptake in VERO cells without sodium fluoride exceeded uptake in CHO cells with sodium fluoride.

Analysis of the slopes of T-2 uptake curves displayed the influence of sodium fluoride on rate of toxin uptake in these cell types. Figures 2 A-D are regression lines of the initial T-2 toxin uptake (0-20 min) at 22 and 37^0 C for both toxin doses. In VERO cells, sodium fluoride significantly increased the rate of toxin uptake at both temperatures and drug concentrations. In CHO cells, this was true at 37^0 C, but at 22^0 C sodium fluoride increased the rate of toxin uptake at only the higher dose.

Cell volumes are summarized in Table 2. VERO to CHO cell volume ratios ranged from 1.96 to 5.09 with a mean of 3.41. Visual observation of cultures with phase contrast microscopy also demonstrated a larger surface area for VERO cells compared to CHO cells.

Results of total cellular RNA content of both cell types are shown in Table 3. The total cellular RNA content of CHO cells was 3.8 times that of VERO cells ($p<0.05$).

DISCUSSION

Previous studies showed that significant ($p<0.05$) differences existed in uptake of T-2 toxin between VERO and CHO cells at a T-2 dose of 0.01 µg/ml (22 and 37° C) but not at higher doses (TRUSAL AND WATIWAT, 1983). From this data, we postulated a concentration-dependent event. That is, if the number of intracellular binding sites for the toxin were significantly different in each cell type, differences in T-2 uptake might be more distinguishable at lower toxin doses. At higher toxin doses (e.g., 0.05, and 1.0 µg/ml), the number of toxin molecules might be in vast excess of the number of intracellular binding sites, therefore masking any cell type differences. We also found that VERO cells consistently displayed a greater inhibition of protein synthesis when compared to CHO cells under identical conditions (TRUSAL, 1985). This greater inhibition of protein synthesis in VERO cells was consistent with noted differences in toxin uptake between the two cell types. This study was designed to investigate cell type differences in T-2 toxin uptake and to study the effect of sodium fluoride on this process.

When toxin uptake was normalized on a molecules per cell basis, significant differences existed in T-2 toxin uptake in VERO and CHO cells pre-incubated with sodium fluoride. In addition, there were also significant differences in toxin upake over time between cell types.

Since T-2 mycotoxin binds to eucaryotic ribosomes (CANNON et al., 1976; HOBDEN and CUNDLIFFE, 1980), differences between toxin uptake in VERO versus CHO cells might be explained by a larger number of intracellular binding sites (i.e., ribosomes) in VERO cells. WATSON (1976) determined that approximately 80% of total cellular RNA is ribosomal. Using scanning microdensitometry, we determined that total RNA content was 3.8 times that of VERO cells. Therefore, the greater T-2 uptake in VERO cells compared to CHO cells could not be explained on the basis of ribosomal binding sites.

MIDDLEBROOK and LEATHERMAN (manuscript in preparation) established that T-2 binds equally well to ribosomes isolated from CHO cells, reticulocytes or yeast and that differences in whole cell uptake cannot be explained solely by differences in ribosomal binding. Our results support this conclusion.

If there are intracellular binding sites other than ribosomes, or a significant amount of toxin remains non-organelle-associated, then the volume of the cell may play a role in total amount of intracellular toxin. Cell volume measurements revealed that VERO cell volume exceeded CHO cell volume by a factor of 3.41 (Table 2). Once toxin enters the cell, it is difficult to speculate what other cellular constitutents might serve as intracellular binding sites, due to the lack of information in the literature. It is possible that diffusion and total cellular volume may play a predominate role in total toxin uptake in the absence of sodium fluoride. In addition, fluoride exposure in HeLa cells has been shown not to affect average cell size or relative distribution of various sized cells (CARLSON AND SUTTIE, 1967) and as such should not have been a factor in cell volume measurements.

Sodium fluoride is known to have a variety of effects on cultured cells, dependent on such

factors as cell type, pH, and growth media composition (HOLLAND, 1980). Sodium fluoride inhibits cell growth and is cytotoxic with prolonged exposure and higher doses (HOLLAND, 1980, TSUTSUI et al., 1984). In cell lines such as mouse fibroblasts, human diploid fibroblasts, HeLa cells and CHO cells, fluoride causes inhibition of protein synthesis. Most workers believe this inhibition is the primary cause of sodium fluoride cytotoxicity (HOLLAND et al., 1980, and MANKOVITZ et al., 1978). In order to be sure that sodium fluoride was not cytotoxic in our system, we exposed CHO and VERO cultures to 5mM sodium fluoride for 4 hr. The data in Table 1 show that sodium fluoride was not cytotoxic to either cell type as assayed by release of LDH. To the contrary, sodium fluoride tended to stabalize the release of residual LDH over time compared to controls (Table 1).

Another action of fluoride was also not relevant to our results. Although T-2 is a potent inhibitor of protein synthesis, this action of the toxin and possible additive effects of sodium fluoride were not relevant, since we did not examine inhibition of protein synthesis in this study.

Fluoride's other major action is it's effect on the glycolytic pathway, although the literature is contradictory about this effect in cultured cells. Two reports indicate that 6 mM sodium fluoride did not inhibit glycolysis in LS cells (HOLLAND and HONGSLO, 1978,1979), while others reported 1.2 mM sodium fluoride decreased glycolysis in human red blood cells and Ehrlich tumor cells (GUMINSKA and STERKIVICZ, 1976). Fluoride exposure also caused an enolase-mediated inhibition of glycolysis (CIMASONI, 1972). Our use of 5 mM sodium fluoride falls within the concentration range reported by other workers. We believe that sodium fluoride's effect on the glycolytic pathway is relevant to increased T-2 toxin uptake or decreased toxin export in our system. It is possible that T-2 toxin's entry or export into or from cells respectively, is an energy dependent process. THOMPSON AND WANNEMACHER (1983) found that T-2 toxin moves rapidly in and out of Vero cells. When toxin was removed from the media, one-half of all the labeled toxin left the cell within 1-2 hr and 90% by 4 hr. MIDDLEBROOK and LEATHERMAN (manuscript in preparation) studied the export of T-2 toxin out of VERO cells with and without the presence of sodium fluoride. Their data established that export of T-2 toxin out of cells was slowed 2 to 5 times in the presence of 10 mM sodium fluoride. Therefore, since sodium fluoride inhibits cellular events dependent on ATP produced by glycolysis, our data imply that inhibition of glycolysis leads to increased cell-associated toxin. We cannot definitively state if this increased cell-associated T-2 toxin is due to increased uptake, retention, or a combination of both events in these cultured cells. Work needs to be done to establish definitively that sodium fluoride's major effect on T-2 toxin uptake is related to its glycolytic energy inhibition. Such work needs to define if increased cell-associated T-2 toxin caused by sodium fluoride is the result of increased uptake, retention or both events.

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Table 1

EFFECT OF SODIUM FLUORIDE ON LACTATE DEHYDROGENASE RELEASE IN CHO AND VERO CELLS

	LDH values*†	
Time (min)	CHO	VERO
No Toxin	42 ± 4.2	34 ± 5.9
0	43 ± 3.2	38 ± 2.0
30	39 ± 3.4	32 ± 4.2
60	33 ± 1.9	43 ± 2.6
90	34 ± 2.8	39 ± 1.4
120	30 ± 1.2	38 ± 4.1
150	30 ± 4.8	40 ± 4.2
180	33 ± 6.4	35 ± 4.2
210	29 ± 7.7	32 ± 5.4
240	36 ± 6.6	31 ± 5.1

* = LDH values represent the mean of two experiments and are expressed in IU/l ± S.E.

† = No time points had significant P-values at $P<0.05$ confidence level by using the one-way analysis of variance test.

Table 2

SUMMARY OF CELL VOLUME DETERMINATIONS OF VERO VERSUS CHO CELLS*

	CHO Volume	VERO Volume	Ratio VERO/CHO
Experiment 1	6.5 ± 0.67	32.9 ± 4.3	5.09
Experiment 2	17.8 ± 3.0	39.9 ± 5.8	2.24
Experiment 3	8.6 ± 0.70	16.8 ± 0.94	1.96
Experiment 4	4.6 ± 0.23	19.9 ± 0.95	4.36
Mean VERO/CHO Volume Ratio ± S.E.			3.41 ± 0.77

* = Values represent mean ± S.E.

Table 3

TOTAL CELLULAR RNA CONTENT OF CONTROL CHO AND VERO CELL POPULATIONS*

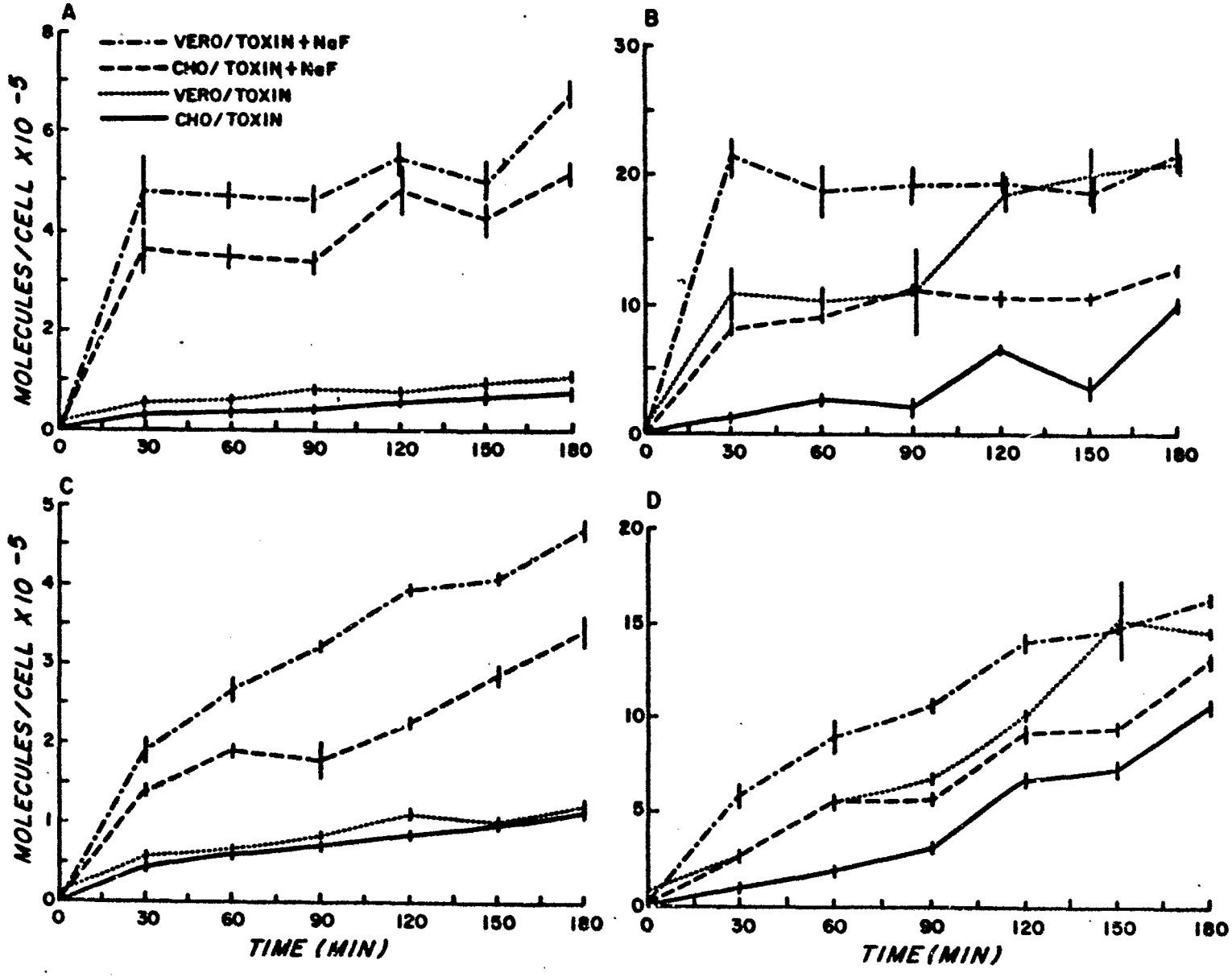
Cell Type	Absorbance Units
CHO	18.60† ± 1.88
VERO	4.94† ± 0.36

* = Values are expressed as absorbance units ± S.E.
† = Significant P-value at $P<0.05$ confidence level by using Duncan's Multiple Range Test

LEGENDS FOR FIGURES

Fig. 1 - Line graph of T-2 toxin uptake in CHO and VERO cells ± sodium fluoride expressed in molecules/cell x 10^{-5} (A): 0.001 µg/ml, 37° C; (B) 0.01 µg/ml, 37° C ; (C) 0.001 µg/ml, 22° C; (D) 0.01 µg/ml, 22° C. Data represent mean of two experiments. Bars represent ± S.E.M.

Fig. 2 - Regression lines of T-2 uptake from 0-20 min at 22 and 37° C expressed in molecules/cell x 10^{-4} (A): CHO, 0.001 µg/ml ; (B) CHO, 0.01 µg/ml ; (C) VERO, 0.001 µg/ml; and (D) VERO, 0.01 µg/ml. Data represent replicate means of one experiment.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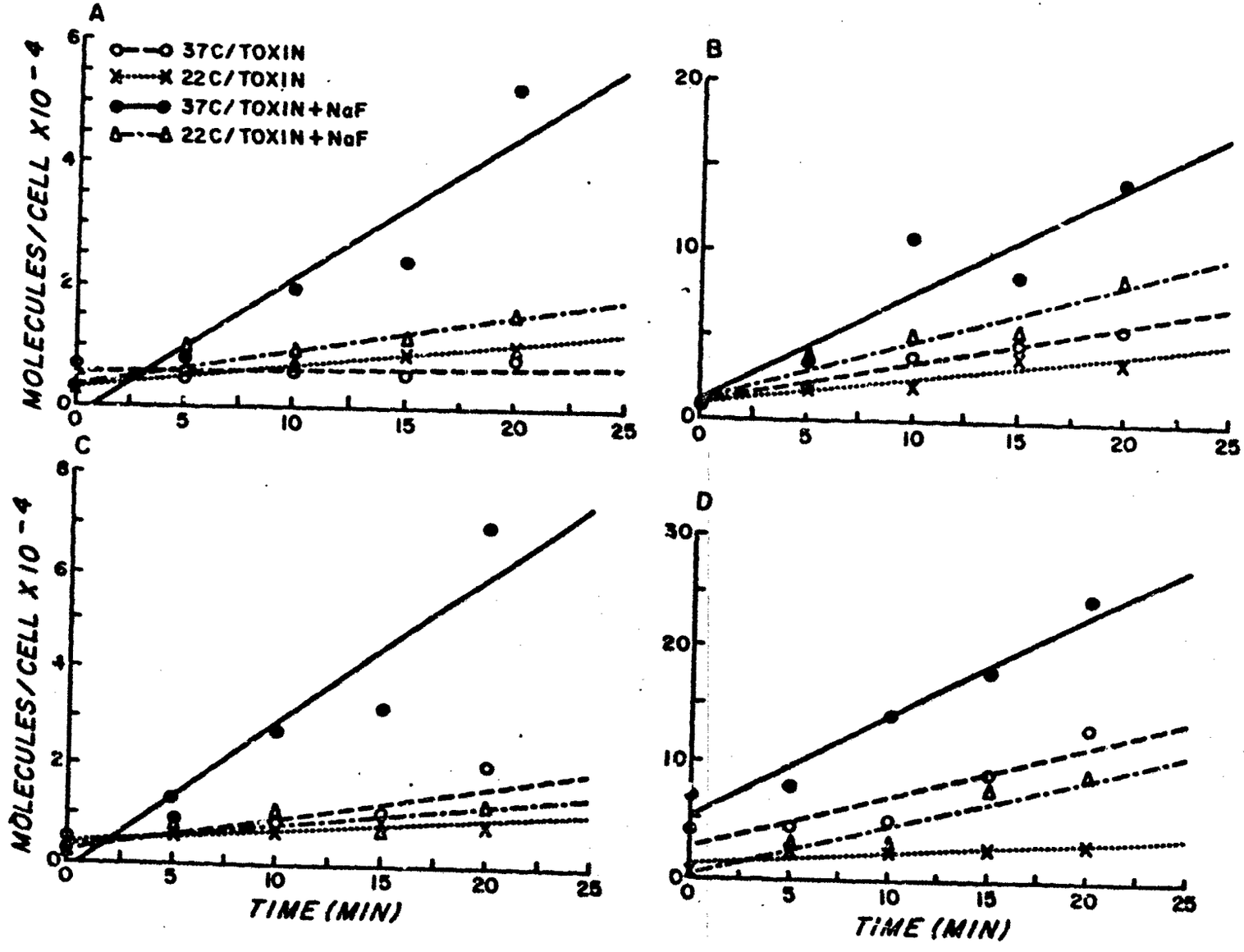
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